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Tissue Responses of *Cyprinodon variegatus* to the Myxosporidian Parasite, *Myxobolus lintoni* Gurley.

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(Plates I-VII).

INTRODUCTION.

Little is known about the histopathology of myxosporidian infections. The majority of these protozoan parasites are found in the gall-bladder and urinary bladder of fresh water and marine fishes but cause little or no damage to the host tissues. However, when the infections become localized in the epithelium of the intestine, in the liver, or in other organs, a considerable number of pathological changes may occur. If the fins, skin or musculature are invaded, the resulting lesions are very conspicuous, producing in many instances tumor-like growths.

One of the best known myxosporidian tumors is found on the European Barbel, *Barbus barbus,* and is called the "boil disease" or "Beulenkrankheit." This condition is caused by *Myxobolus pfeifferi* Thélohan and has been reported for other European fresh water fishes such as *Barbus fluviatilis* and *B. plebejus.* Keysellitz (1908) has found tumors on these fish varying in size from that of a millet grain to one as big as a hen's egg, and as many as twenty-three small ones on a single fish. According to Doflein (1928), the causative agent may be found in all organs of the body. When the musculature becomes infected tumor-like growths are formed. Microscopically, such structures show a hypertrophy of the "interfibrillares" connective tissues, Myxosporidia, degenerated muscle, and a secondary infection with bacteria.

Fiebiger and Kahls (1929) have also described the "boil disease" from certain Austrian fishes. In the Barbel, the typical nodules were found, showing the characteristic pathological picture. However, a similar infection in the Giant Perch (*Lucioperca sandra*) resulted in no pathological changes, except for some vascular congestion.

Plehn (1910) reported that *Myxobolus piriformis* Thélohan produced in a European minnow, *Leuciscus,* growths which resemble a papillary adenocystoma, but which were not regarded as true tumors. These are usually found in the tail and histologically show folds of the epithelium of the skin with the parasites within the folds.

Hahn (1913) described tumors from the common killifish, *Fundulus*

heteroclitus, resulting from an infection of *Myxobolus musculi* = *M. funduli* Kudo, 1919. The lesions, according to Hahn, are caused primarily by worm parasites or mechanical injury and only secondarily do they become infected with Myxosporidia. Microscopically, the tumors show an infiltration with small lymphocytes and a preponderance of vascular tissue and erythrocytes. Numerous non-staining granules of unknown nature and origin were found in infected epidermis, in connective tissue of the dermis and in atrophied muscle fibers. The latter were completely degenerated, showing but few fibers with normal fibrillae and cross striations. The entire tumor mass eventually becomes infected with bacteria. According to Hahn, the vegetative stages of the parasite are intracellular and found within the muscle fibers.

Kudo (1929, 1931) has noted tumor formation due to *Myxobolus* and related Myxosporidia in several species of fresh water fishes. One form, *Myxobolus notatus* Mavor, is found in the sub-dermal connective tissues of the tail muscles of the blunt-nosed minnow, *Pimephales notatus* (*Hyborhynchus notatus*) and *Leuciscus rutilis*. The tumor enclosing a trophozoite reaches a diameter of 7 mm. Histologically, the growths are composed of large cysts which are surrounded by the sub-dermal cells of the hosts. The tissue around the parasite becomes so highly changed that it appears as an "epithelium." The tissues not in contact with the cysts show no cytological changes. However, such areas have an abundance of blood capillaries. The nuclei of the cells in direct contact with the cysts become hypertrophied; the cells stain deeper than the normal cells, and lack the distinct membrane characteristic of epithelial cells. They vary considerably in size and shape, being columnar, club-shaped or irregularly rounded, and do not form a continuous layer. Kudo believed that these "epithelial cells" are modified connective tissue cells. Similar pathological pictures were described by Mavor (1916) and Debaisieux (1925).

There are other genera of Myxosporidia that produce definite responses of host tissues. In some cases such infections result in a complete degeneration of the invaded areas, often causing the death of the fish; other infections result in a simple hypertrophy of the connective tissue cells, enclosing the spores or vegetative stages of the parasite (Plehn, 1905; Kudo, 1919, 1926; Davis, 1923; Dunkerly, 1925).

Recently, the writers have encountered several specimens of *Cyprinodon variegatus* Lacépède with large myxosporidial tumors of the body. This has afforded the opportunity of making a study of various histological responses of the host resulting from such an infection. It is recalled that this disease was first noted by Linton (1889) in sheepshead minnows caught at Woods Hole, Massachusetts. Gurley (1891) definitely allocated the parasite causing the tumors and named it *Myxobolus lintoni*. In 1894, he gave a more detailed description of the species. Similar parasites and tumors were observed by Hahn (1913).

Material and Methods.

The infected *Cyprinodon* were caught at Sandy Hook Bay, New Jersey, and the Connecticut River. The tissues were fixed in 10% neutral formalin, and embedded in paraffin after decalcification. These were then sectioned at 3-10 μ thick and stained with Giemsa's, Wright's, hematoxylin-eosin and Masson's special connective tissue stain. An examination of the spores in these preparations showed them to be *Myxobolus lintoni* Gurley.

Description of the Parasites.

Vegetative Stages. Histological examination of the tumors revealed many stages in the development of the parasite. Although an occasional

spore was found free in the lumen of the intestine, there was no evidence of sporoplasm or multiplicative stages in the gut-epithelium. The tumors themselves are composed of enormous numbers of spores and vegetative parasitic masses surrounded by host tissues (Figs. 4-7). These masses vary as to number. Thus in certain flat tumors (Fig. 1), there are only a few present and more or less widely scattered. In more rounded pendant types of tumors (Figs. 2 & 3), the vegetative groups are numerous but are situated mostly at the base or the periphery near the body wall. The parasites towards the surface of the growths are usually fully developed spores. The size and shape of the circumscribed vegetative masses also vary a great deal. A few are spherical, measuring from 30 μ to 66 μ in diameter. The majority of them, however, are oval and range in size from 18 x 29 to 62 x 128.7 μ.

Each vegetative mass demonstrates clearly various stages in sporogenesis. In the larger groups may be found sporonts with two, four and six nuclei and minute basophilic staining granules in the cytoplasm, or young spores with a nucleus at the base of each polar capsule and two or four nuclei in the sporoplasm, depending upon whether or not the shell is developed. In these young spores, the polar filaments are in the form of basophilic granules aligned spirally. Scattered throughout the vegetative masses may be found nuclei of different forms together with degenerating host tissue. Other large circumscribed masses contain only matured spores (Fig. 7), many with but a single nucleus in the sporoplasm. In smaller masses (10-15 μ in diameter) trophozoites with many nuclei are present. According to Kudo, and other investigators, the larger of the nuclei are vegetative, while the smaller become generative nuclei. The latter are "budded" off with a small bit of cytoplasm and become the sporonts. The nucleus of the sporont divides several times until, in this species at least, six nuclei are produced. Such cells have basophilic granules in the cytoplasm. These may be discarded nucleoli; a phenomenon previously reported for other Myxosporidia (see Kudo, 1926). In so far as could be determined, the sporont at this stage becomes transformed into the definitive spore. It is assumed that in the final stage of sporogenesis two nuclei are used in the formation of the polar capsule, two for the shell and two remain as the nuclei of the sporoplasm. Therefore, unlike the majority of forms belonging to the genus *Myxobolus* the pansporoblast of *M. lintoni* gives rise to a single spore, instead of two or more.

Spore. The present observations on the fully developed spores agree with those of Linton and Gurley, except in size. The measurements given by Gurley and also used by Kudo (1919) are as follows: length 13.9 μ; breadth 11 μ; thickness 8 μ. Certain of our measurements agree with those given by Gurley, but the average size, for over 200 spores taken at random, is slightly smaller than those given above, measuring 10.96 x 7.47 x 6.46 μ. The extreme measurements are as follows: length 9.13 to 14.9; width 4.98 to 10.40; thickness 4.15 to 7.47 μ. However, the smaller size recorded here for *Myxobolus lintoni* is no doubt partly due to shrinkage following fixation.

The spores (Fig. 7) are oval in shape. The shell is more or less thick and with a fairly marked sutural ridge. There are two polar capsules, having an average measurement of 4 μ in length and 2 μ in width. The sporoplasm usually contains two nuclei and a large "iodinophilous" vacuole. The more mature spores have but a single nucleus which, according to Kudo (1931), is the result of a fusion of the two nuclei of the younger spore.

With Masson's stain the shell, capsule and vacuole of the sporoplasm are colored green; the polar filaments and sporoplasm are colored red; while the nuclei take on a deeper red color.

Although the organisms are usually localized in the tumor mass, an occasional spore was found in the lumen of the intestine, on the gills, in the liver, kidney and external to the meninges of the cord. None of these spores resemble the species reported by Davis (1917) as *Myxobolus*

capsulatus which was found in the visceral connective tissues of *Cyprinodon* from Beaufort, North Carolina. According to this investigator, *M. capsulatus* is present in a state of diffuse infiltration, and differs from *M. lintoni* mainly in having longer polar capsules, almost two-thirds the length of the spore.

Description of the Tumors.

As was previously mentioned, two types of tumors were encountered. Fig. 1 shows the flattened type with the overlying skin slightly ulcerating and pigmented. Figs. 2 and 3 demonstrate the large pendant types of tumors. The fish in Fig. 2 measures about 4 cm. in length. The tumor is situated on the dorsal surface of the body and measures 8 x 10 mm., the upper border extending above the surface of the body. The fish in Fig. 3 is 4.24 cm. in length and the tumor, irregular in shape, measures approximately 1 x 1.2 cm. This is situated behind the left pectoral fin and extends dorsally for about one-half the body width. The ventral border of the tumor reaches about 4 mm. below the surface of the body. In the last two fish, the skin appears to be sloughed off, while the tumor of the fish in Fig. 3, is beginning to show signs of ulceration.

In most of the microscopic preparations the loss of the epithelium covering the surface of the growths is apparent. The external boundary of the tumors consists of a single layer of elongated flattened melanophores (Fig. 6, p). These pigment cells are of the usual corial type found in this fish, normally lying under the transparent epithelium. It is immediately below this narrow outer layer of pigment cells that characteristic changes of host tissue occur (Figs. 4-15). The diffusely scattered spores and vegetative masses of varying sizes are embedded in a meshwork of delicate fibroblasts of the host. As was previously mentioned, the diffuse arrangement of the spores is more frequently encountered near the free surface of the tumor, whereas the circumscribed collections of spores or vegetative stages are more numerous near the base or the mesial aspect of the tumor. It is in the deeper parts of the growth that the fibroblastic response of the host is most marked. Many delicate spindle-shaped, irregular or often stellate forms of fibroblasts are loosely arranged between the parasitic masses forming a stroma of the tumor which separates and supports the circumscribed collections of the organisms (Fig. 6, fi). It is only rarely that the connective tissue stroma takes on a denser appearance (Fig. 8). Lying in the stroma are occasional polynuclear cells, lymphocytes, eosinophiles and mast cells. This leucocytic inflammatory reaction is a very mild one, and there is practically no evidence of necrosis. The connective tissue stroma contains also a few melanophores which perhaps have migrated into the mass of Myxosporidia (Figs. 8 and 10, p). Bacteria, bacilli and a few cocci, may be seen near the surface of some of the myxosporidial tumors. These doubtless represent a secondary infection.

In most microscopic fields there co-exists with the fibrous stroma, a serous or albuminous substance staining faintly pinkish with eosin in which Myxosporidia lie scattered (Fig. 9).

The vascular supply of myxosporidial growths consists of a very fine network of capillary blood vessels, very often only wide enough to permit the passage of a single file of red blood cells. In some fields a few erythrocytes lie in the tissue spaces outside the lumen of the capillaries. These cells are normal in appearance, and indicate a mild form of extravasation of blood. No hemorrhages of importance were noted. A few small nerve trunks were found in areas involved in myxosporidial infection, but these did not seem to be affected by the diseased conditions.

The tumors of *Cyprinodon* are not encapsulated, as an irregular infiltration by the organisms into adjacent structures occurs along the margins of

these growths. Myxosporidia gain access to the deeper lying muscular structure by penetrating through the relatively dense external limiting fascia which normally separates muscles from the layers of the skin (Fig. 10, f). The fibers of this fascial structure become fragmented and split in the longitudinal direction into finer bundles between which Myxosporidia in small groups are found. Organisms which collect below the level of the external limiting membrane spread out along the fibrous septa which separates muscle bundles. This streaming infiltration along intermuscular septa is shown in Fig. 11. In some places the vegetative masses were found well organized in the fibrous tissue separating groups of muscle fibers (Fig. 12). Where the diseased process is advanced, atrophy, loss of striation, hyalin degeneration and necrosis of muscle fibers may occur (Fig. 12, n). In the sections examined we have found no evidence of spores or vegetative stages lying directly in the individual muscle cells as reported by Hahn (1913).

In microscopic sections prepared in the region of the body cavity of one fish, there were found certain areas where a penetration of organisms through the pigmented peritoneal lining had occurred (Fig. 13). This was usually only a slight involvement of the peritoneum, and interestingly enough, was not associated with an inflammatory exudate. The normally densely massed pigmented cells of the peritoneum were disarranged so that groups of melanophores were seen separated by collections of Myxosporidia. Although this penetration of myxosporidial tumor had occurred immediately in the region of the liver, an infiltration of liver substance had not taken place to any extent, as only a very few spores could be identified in the liver tissue.

Partial destruction of scales in the diseased regions was not uncommon. Bony tissue and cartilage, when lying in a myxosporidial growth, were found surrounded and infiltrated by spores (Fig. 14). This was particularly true in the case of spongy bone. where the meshes between the bony spicules were filled with varying numbers of spores (Fig. 15). Here and there the bony spicules appeared degenerated and necrotic.

Discussion.

Microscopic tissue changes caused by myxosporidial infections have been described by previous investigators, and have been referred to in the introduction. The present studies are restricted to material obtained from Sandy Hook Bay, New Jersey, and the mouth of the Connecticut River. These fish were caught during August and the early part of September, 1937, in the two locations about 100 miles apart. The lesions in the fish from both localities were similarly advanced and much the same in character and distribution. It became apparent, after microscopic studies, that there was a mild general infestation in each fish examined, as indicated by the occurrence of spores in the liver, kidney, on the gills and in the lumen of the intestine. In these organs no histological lesions were evoked. Contrasting with this, the skin and subcutaneous lesions were most extensive, as there resulted in these regions relatively large tumor masses. Here the Myxosporidia were seen as diffuse spores or as circumscribed vegetative masses in various phases of development and embedded in a delicate meshwork of fibroblastic tissue varying somewhat in density. This meshwork of fibroblasts, with its rich capillary blood supply, represents one of the major responses of the host to the myxosporidial infection. Associated with it there occurs a wide-spread exudate which stains a light pinkish color with eosin and thus resembles a serous or albuminous material. In certain areas, the exudate exists as a homogenous substance in which spores and vegetative masses lie free. In other places, it is granular or appears to contain fine threads not unlike fibrin. The exudate accompanies fibrous tissue organization. It is least conspicuous where the fibro-

blastic network is most highly developed and condensed. The fact that the exudate is present at sites without fibroblastic development suggests the possibility that it precedes the fibrous organization of the tumors.

The spread of the myxosporidial parasites from the subcutaneous region to the muscles of the body wall is accomplished by their passing between the loosened fibers of the external limiting fascia. Intermuscular fibrous septa form pathways for the parasites to reach the deep seated musculature and the peritoneum. In one specimen a limited involvement of the peritoneum had occurred.

Myxosporidia infecting intermuscular fascia may result in the degeneration of muscle tissue itself. This is evidenced by the loss of striation, hyalinization and atrophy of the fibers.

Bony and cartilaginous structures, interposed in the spread of the infection, are surrounded and infiltrated by spores and may become necrotic.

A secondary invasion of bacteria, which frequently occurs, may contribute to bring about destruction of various tissues infected by Myxosporidia.

Summary.

1. Several specimens of *Cyprinodon variegatus* were found showing tumors caused by the Myxosporidian, *Myxobolus lintoni* Gurley.

2. Certain stages in sporogenesis are reported and the morphology of the spore redescribed.

3. In so far as could be determined, the sporont of *M. lintoni* gives rise to a single spore.

4. This sporont has six nuclei and several discarded nucleoli in the cytoplasm. As in many Myxosporidia, two of the nuclei probably give rise to the polar capsules, two to the shell and two remain as the nuclei of the sporoplasm.

5. With Masson's special connective tissue stain the shell, capsule and vacuole of the sporoplasm are colored green; the polar filaments and sporoplasm are colored red; while the nuclei take on a deeper red color.

6. Various histological changes in the host tissue due to the infection with *Myxobolus lintoni* are described.

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EXPLANATION OF THE PLATES.

Plate I.

Fig. 1. Live *Cyprinodon* taken at Sandy Hook, N. J. This fish shows flat myxosporidian tumors.

Figs. 2 & 3. *Cyprinodon* with large pendant types of myxosporidian tumors. Killed and fixed specimens taken from Sandy Hook.

Plate II.

Fig. 4. Low power photomicrograph through the myxosporidian tumor of the skin. x 40. Stained with Giemsa.

Fig. 5. A slightly higher magnification of the tumor shown in Fig. 4. Note the vegetative parasitic masses in the deeper parts of the tumor. x 75.

Plate III.

Fig. 6. Tumor showing circumscribed vegetative parasites and spores. Note the fine network of fibroblasts (fi) between the parasitic masses. Outer surface lined by pigment cells (p); the epidermis has disappeared. About x 250.

Fig. 7. Fully developed spores of *Myxobolus lintoni.* x 1500.

Plate IV.

Fig. 8. Fibroblastic respose of the host supporting vegetative, parasitic masses. Note that in this section the fibroblasts (fi) are denser than those shown in Fig. 6. A large single melanophore (p) with its dendrites may be seen in the center. No albuminous exudate present. x 375.

Fig. 9. Section showing a single vegetative mass with developing spores. A few solitary spores are present in the adjacent albuminous or serous exudate. About x 500.

Plate V.

Fig. 10. Low power photomicrograph showing extension of the myxosporidian parasites through the loosened external limiting fascia (f). (m), muscle layer. (p), pigment cells. x 90.

Fig. 11. Section showing spores scattered along the intermuscular fibrous tissue. (m) muscle. x 375.

Plate VI.

Fig. 12. Section showing infiltration of intermuscular connective tissue by the myxosporidian parasites. (n) necrotic muscle fibers. x 90.

Fig. 13. Fragmentation of the pigmented peritoneum by Myxosporidia. About x 375.

Plate VII.

Fig. 14. Myxosporidia surrounding bony spicules (b). x 100.

Fig. 15. Infiltration of spongy bone by the spores of *Myxobolus lintoni.* x 750.